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A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

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ROGER FRANKLIN VARNEY

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A METHOD FOR THE ASSAY OF THE GONADOTROPIN CONTENT OF NORMAL HUMAN URINE[1]

ROGER F. VARNEY

From the Department of Biochemistry, University of Chicago
CHICAGO, ILLINOIS

THE PURPOSE OF THE WORK reported here is to establish a reliable, simple, reproducible assay method for gonadotropic activity in the urine from normal men and women. Among the many methods which have been suggested for this purpose, the following should be emphasized. Zondek's (1) alcohol precipitation has been modified and used by Albright, Halsted and Cloney (2), Saethre (3), Heller and Heller (4), McCullagh (5), and many others. Katzman originated the use of tungstic acid (6, 7, 8) and the adsorption of the activity on finely divided benzoic acid (8, 9). The latter he later abandoned (10). Levin and Tyndale (11, 12, 13) favor precipitation by tannic acid, a method also used by Katzman (10) Friedman and Weinstein (14) and von Haam and Rothermich (15).

In preliminary work we found the tannic acid method satisfactory for human pregnancy urine, but when we attempted to use it on the much less active urine of normal women, we found that it was useless because of the high toxicity of the tannic acid precipitates. Our experience with the benzoic acid adsorption method of Katzman confirms his later conclusions (10), namely, that in this procedure there is considerable loss of gonadotropic activity (see table 1). In many cases the benzoic acid precipitates from urines of low activity were too toxic to permit assay. The alcohol-ether method originated by Zondek (1) as modified by Leonard and Smith (16) was also studied.

Our reasons for choosing the mouse as the assay animal, as explained in detail in other papers (17, 18), are based on the much greater sensitivity of the mouse uterus to the active substance and the greater uniformity of the response to increasing doses of the material. Our mouse unit is defined as the amount of activity necessary to cause a 100 per cent increase over the controls in the uterine weight of an immature (6–8 gm.) white female mouse. The material to be assayed is dissolved or suspended in 3 cc. of saline and 0.5 cc. is injected subcutaneously twice a day for three days. The test animals are killed on the morning of the fifth day, the uteri removed, freed of connective tissue and contained fluid and weighed on a torsion balance. Four or more mice are used on each dosage level. The levels injected are calculated so that a positive response in each successive dosage group of animals corresponds to 10, 20, and 30 mouse uterine units, respectively, per 24-hour samples of urines from normal males and females. In urines in which higher activity is expected the dilution is increased proportionately. This method parallels closely the mouse uterine method of Levin and Tyndale (12), but there are some differences in that they use somewhat larger mice (7–10 gm.), inject once daily for three days, and autopsy 72 hours after the first injection. Their mouse uterine unit is defined as the amount of activity causing

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a 100 to 150 per cent increase over the control uterine weight. In spite of these differences in technic, an assay by us with a tannate sent by Dr. Levin shows that our assay agrees with his within 25 per cent and values obtained in the two laboratories on various types of urine seem to indicate that the units are approximately the same.

EXPERIMENTAL PROCEDURE

Preparation of alcohol precipitate. To the urine which was chilled and adjusted to pH 4.8 to 5.2 with acetic acid, four volumes of 95 per cent ethanol were added. The 'crude alcohol precipitate' was collected by settling and centrifugation in the freezing room. In urines from postmenopausal or castrate women in which the activity is relatively high, this crude precipitate can be injected without further treatment, but for assays on less active urines toxic substances must be removed. The results recorded in this paper clearly show the preference of the 50 per cent alcohol extraction procedure applied on this crude alcohol precipitate over the dialysis thereof and over the tannic acid precipitation process.

Recovery of added activity from urine of normal males. This portion of the work tests the recovery from urines of normal males of added active material from the

TABLE 1. RECOVERY OF ADDED GONADOTROPIN[1] FROM URINE OF NORMAL MALES BY THE ALCOHOL AND BENZOIC ACID METHODS

Aliquots and Treatment	Mouse Uterine Units per Liter	Total Mouse Uterine Units
A. Normal male urine (5 liters) precipitated with 4 volumes of ethanol. No added gonadotropin	>8	>40
B. Normal male urine (5 liters) with 133 m.u.u.[2] gonadotropin added. Precipitated with 4 volumes of ethanol	>40	>200
C. Normal male urine (5 liters) with 133 m.u.u.[2] gonadotropin added. Precipitated by benzoic acid method	2.5–5.0	12.5–25.0
D. Equivalent of 1 liter of the supernatant from *aliquot* C precipitated with 4 volumes ethanol	20	100

[1] We wish to thank Dr. J. A. Morrell of E. R. Squibb & Sons, New Brunswick, N.J., for this material (Gamone).

[2] Assayed previously by us on mice.

urine[2] of postmenopausal women by the benzoic acid method and by the alcohol method. The alcohol and benzoic acid precipitates were assayed without further treatment since the high activity makes the toxicity a negligible factor. From table 1 it is seen that the alcohol method recovers more than 200 mouse uterine units compared to the 12 to 25 M.U.U. recovered by the benzoic acid method. That the activity which is not present in the benzoic acid precipitate is partially destroyed is indicated by the fact that of the more than 200 M.U.U. shown to be present (*aliquot B*), only 12 to 25 M.U.U. are recovered in the benzoic acid precipitate (*aliquot* C) and 100 units by the subsequent alcohol precipitation of the supernatant from the benzoic acid precipitation (*aliquot D*). Thus of more than 200 M.U.U. present not more than 125 can be recovered by the consecutive application of the benzoic acid and alcohol methods on the same solution, indicating that not only does the benzoic acid method fail to recover all of the activity, but that some of it is lost or altered so that it cannot be recovered by subsequent alcohol precipitation. This confirms the findings of Katzman (10).

Efficacy of alcohol precipitation. Losses in dialysis. Since in urines of low gonado-

[2] Postmeuopausal urine activity (Gamone) concentrated by E. R. Squibb and Sons, New Brunswick, N. J.

tropic potency, the crude alcohol precipitate is too toxic to permit assay, dialysis was used in an attempt to reduce the toxicity. The dry alcohol precipitate was extracted three times with water, the combined aqueous extracts dialyzed in Viscose tubing overnight against running tap water and the activity then precipitated by the addition of 4 volumes of acetone and a trace of sodium acetate. This precipitate was suspended in saline and aliquots taken for injection. This procedure is the same as that used by Heller and Heller (4) but was developed independently. Our studies on the magnitude, cause, and prevention of losses in this process are of some interest. Table 2 shows the loss caused by the dialysis procedure as outlined above when it is applied to a pooled crude alcohol precipitate from postmenopausal urine. It is obvious from table 2 that the loss of 47 per cent of the activity may be due to extraction, dialysis, or acetone reprecipitation.

TABLE 2. LOSS OF ACTIVITY OF POSTMENOPAUSAL URINE DURING DIALYSIS

Aliquot and Treatment	M.U.U./day
A. Aqueous extract of crude ethanol precipitate dialyzed and reprecipitated with 4 volumes of acetone	200
B. Crude alcohol precipitate suspended in water	375

Table 3 shows the results of an attempt to localize the loss with respect to the three steps involved in the above treatment. The same pooled alcohol precipitate from postmenopausal urine was used. The data in this table indicate that the loss takes place entirely during dialysis. Attempts to decrease this loss by changing the conditions of dialysis were unsuccessful.

TABLE 3. LOSSES DUE TO THE THREE STEPS OF THE DIALYSIS PROCEDURE

Aliquot and Treatment	M.U.U./day
A. Alcohol precipitate suspended in water	450
B. Aqueous extract of alcohol precipitate	500
C. Dialyzed aqueous extract of alcohol precipitate	250
D. Dialyzed aqueous extract of alcohol precipitate reprecipitated with 4 volumes of acetone	250

A comparison of dialysis losses with the total activity present in a sample of postmenopausal urine is shown in table 4. In this experiment the entire sample of urine was extracted with n-butanol and ether to remove combined and free estrogens which interfere in the assay. The aqueous phase was treated as indicated. The yield by the alcohol method with dialysis is somewhat less than 50 per cent of the activity of the raw urine. That most of the loss is due to dialysis itself is shown by the fact that *aliquot B* which was dialyzed alone yields only 60 per cent of the raw urine's activity. This confirms the previous evidence that more than 75 per cent of

TABLE 4. COMPARISON OF ACTIVITY OF RAW POSTMENOPAUSAL URINE WITH CONCENTRATES PREPARED BY DIFFERENT METHODS

Aliquot and Treatment	M.U.U.
A. Precipitated with 4 vol. ethanol, extracted with water, dialyzed, reprecipitated with 4 vol. acetone	200–250
B. Untreated urine dialyzed	250–350
C. Untreated urine injected directly	450–550
D. Urine dialyzed and precipitated with 4 vol. ethanol	250
E. Activity precipitated with tannic acid; precipitate suspended and injected	toxic

the loss encountered in dialysis of the aqueous extract of the crude alcohol precipitate with subsequent precipitation by acetone is due to the dialysis itself.

Studies on the tannic acid precipitation of gonadotropin. The method as we used it was given to us by Dr. Levin in a personal communication, but has since been published in detail (13). Dr. Levin also sent us some tannate prepared by him from urine of normal males. This material when submitted to the alkaline decomposition recommended by him and assayed by us gave results which agreed very closely with his. This indicates that our alkaline treatment of the powder was efficient and did not cause loss of activity, and that our assay method gives results essentially the same as his. Since tannate precipitates were toxic in our hands, and since the tannic acid we used was not the same grade as that used by Levin, we injected the two tannic acids alone into mice and found that the lowest total dose used (0.5 mg.) spread over three days caused death in all the animals. This occurred somewhat sooner in the animals receiving our tannic acid, indicating that it was more toxic. When tannic acid is injected in combination with one M.U.U. of activity from postmenopausal urine, there is no lessening of tannic acid toxicity. Doses of 0.5 ,0.3, and 0.1 mg. of tannic acid per three days caused death in all animals at approximately the same time whether injected alone or mixed with the gonadotropin. This indicates that there is no lessening of toxicity in the mixture compared to the tannic acid alone as might be expected if a complex were formed between the two, thus lessening the amount of free tannic acid present to act as toxic agent.

TABLE 5. MOUSE UTERINE WEIGHT RESPONSE TO TANNIC ACID PRECIPITATE OF AQUEOUS EXTRACT OF CRUDE ALCOHOL PRECIPITATE COMPARED TO RESPONSE TO INJECTION OF UNTREATED EXTRACT

Treatment	Dose in mg. equivalents of alcohol powder	Average uterine weight
		mg.
Tannate precipitate of aqueous extract of alcohol preciptiate	13.8	5.1
	20.7	8.1
	26.7	24.6
Aqueous extract of alcohol precipitate	13.8	34.8
Control		4.1

In an effort to evaluate the completeness of precipitation of activity by the tannic acid method, two experiments were made. In the first an aqueous extract was prepared from an alcohol precipitate of postmenopausal urine and divided into two aliquots. One was injected without further treatment and the other was precipitated with tannic acid by the method of Levin including the alkaline decomposition of the tannate before injection. The results appear in table 5.

It is evident that under these conditions the tannate precipitate contained less than 50 per cent of the total activity present in the starting material. If raw postmenopausal urine after extraction with butanol and ether was used as the source of activity, the tannic acid precipitate contained less than 25 per cent of the activity present in the starting material.

Efficacy of the 50 per cent alcohol extraction of the crude alcohol precipitate to replace dialysis. Preliminary attempts to purify the crude alcohol precipitate from urine of normal males by the method of Gurin, Bachman, and Wilson (20) showed that there was a yield of more than 50 per cent of the activity with an 8.5-fold concentration of gonadotropin and a great reduction in the toxicity.

In view of the encouraging nature of these results it was decided to study the substitution of the 50 per cent ethanol extraction for the dialysis procedure on alcohol precipitates from normal urines. The crude alcohol precipitate from normal males was treated as follows.

Aliquot 1. Fifty per cent ethanol extract of the crude alcohol precipitate reprecipitated with two volumes of ethanol.

Aliquot 2. Aqueous extract of crude alcohol precipitate, dialyzed, and reprecipitated with 4 volumes of acetone.

Aliquot 3. Aqueous extract of crude alcohol precipitate, dialyzed and reprecipitated with 4 volumes of acetone. This precipitate was then extracted with 50 per cent ethanol and reprecipitated with 4 volumes of ethanol.

Aliquot 4. Fifty per cent ethanol extract of the crude alcohol precipitate, reprecipitated with 4 volumes of ethanol.

TABLE 6. RESPONSES TO PREPARATIONS MADE BY 50 PER CENT ETHANOL EXTRACTION OF CRUDE PRECIPITATE COMPARED TO THOSE MADE BY DIALYSIS

	Immature mouse uterine weight response to following dilutions				
	A	B	C	D	E
M.U.U. equivalents	0.50	0.75	1.0	1.5	2.0
Aliquot 1	6.4 (3)	7.0 (3)	14.7 (3)	10.2 (3)	10.3 (3)
Aliquot 2	6.7 (3)	8.3 (2)	10.9 (2)	Toxic	Toxic
Aliquot 3	Toxic	6.7 (3)	8.3 (2)	12.6 (4)	27.9 (2)
Aliquot 4	5.2 (4)	6.8 (4)	11.6 (4)	15.4 (4)	34.0 (2)

Four mice were used on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

TABLE 7. REPETITION OF WORK IN TABLE 6, USING MICE PER LEVEL

	Immature mouse uterine weight response to following dilutions				
	A	B	C	D	E
M.U.U. equivalents	0.50	0.75	1.0	1.5	2.0
Aliquot 1	7.8 (8)	9.5 (8)	18.7 (8)	30.3 (8)	32.1 (8)
Aliquot 2	7.1 (8)	6.8 (3)	14.0 (3)	34.0 (1)	Toxic
Aliquot 3	7.2 (7)	11.1 (7)	22.6 (3)	36.7 (2)	19.5 (5)
Aliquot 4	7.1 (8)	11.5 (8)	25.9 (8)	34.5 (8)	30.0 (5)

Eight mice were used on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

In the alcohol extraction process, since the pH of the 50 per cent alcohol mixture was difficult to measure with the glass electrode, the adjustment was made by means of external indicators to pH 5.4 to 6.8. The results are shown in table 6. Inspection of the table shows that the alcohol method is better than the dialysis method from the standpoint of recovery as well as from that of reduction of toxicity. Combination of the routine water extraction, dialysis and acetone precipitation followed by 50 per cent ethanol extraction and reprecipitation with 4 volumes of alcohol (*aliquot 3*) gives a better recovery than the water extraction and dialysis alone (*aliquot 2*) but not as good as the 50 per cent alcohol extraction alone (*aliquots 1* and *4*). Repetition of this experiment using 8 mice on each level and keeping the volumes as low as possible gave the results shown in table 7. The data show that recovery by the 50 per cent alcohol extraction followed by precipitation with 4 volumes of alcohol (*aliquot 4*) is better than by any other method. Next best is the combination of routine water extraction followed by 50 per cent alcohol extraction of the acetone precipitate from the dialyzed water extract (*aliquot 3*).

Confirmatory results are given in table 8 in which we show the high toxicity of the 50 per cent alcohol insoluble fraction and of the crude alcohol precipitate from urine of normal males. The aliquots recorded in table 8 were prepared as follows.

Aliquot 1. Fifty per cent ethanol extract of crude preparation reprecipitated by 4 volumes of ethanol.

Aliquot 2. Residue from *aliquot 1* (not soluble in 50 per cent ethanol) extracted with water and precipitated with 4 volumes of acetone.

Aliquot 3. Aqueous extract of crude alcohol precipitate dialyzed and precipitated with 4 volumes of acetone.

Aliquot 4. Aqueous extract of crude alcohol precipitate dialyzed and precipitated with 4 volumes of acetone. This precipitate was then extracted with 50 per cent ethanol and reprecipitated by 4 volumes of ethanol.

Aliquot 5. Original crude alcohol precipitate suspended, untreated.

In table 8 comparison of *aliquots 1*, *2*, and *5* shows an excellent separation of the toxic and gonadotropic factors. *Aliquot 1* compared to *aliquot 3* at the 1.0 and 0.75

TABLE 8. RESPONSE TO 50 PER CENT ALCOHOL EXTRACTS COMPARED TO AQUEOUS EXTRACTS AND TO RESIDUE AFTER ALCOHOL EXTRACTION

	Immature mouse uterine weight response to following dilutions			
M.U.U. equivalents	A 0.75	B 1.0	C 1.5	D 2.0
Aliquot 1	13.2 (7)	16.1 (6)	21.8 (7)	22.9 (7)
Aliquot 2	6.7 (3)	Toxic	Toxic	Toxic
Aliquot 3	10.1 (7)	12.2 (8)	21.3 (5)	21.9 (2)
Aliquot 4	8.4 (7)	17.5 (7)	23.0 (8)	27.1 (8)
Aliquot 5	Toxic	Toxic	Toxic	Toxic

Eight mice were started on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

M.U.U. levels shows that the final product of the 50 per cent ethanol extraction (*aliquot 1*) contains about one-third more activity than was obtained by water extraction followed by dialysis (*aliquot 3*). From results on *aliquots 1* and *4* we conclude that the alcohol extraction of the acetone precipitate of the aqueous extract subsequent to dialysis causes no appreciable additional loss in activity, but a marked lowering of toxicity. This observation is good confirmatory evidence for the completeness of the alcohol extraction and also clearly shows that removal of toxicity is more efficient by the 50 per cent alcohol extraction than by dialysis, since even after dialysis additional toxic material is removed by the alcohol extraction procedure.

Another experiment designed to characterize the toxic fraction of urine from normal males from the active material confirms the value of the 50 per cent alcohol procedure. Starting from the crude alcohol precipitate (*fraction 1*) four additional fractions were prepared as follows.

Fraction 2. Fifty per cent ethanol extract of crude alcohol precipitate reprecipitated with 4 volumes of alcohol.

Fraction 3. Aqueous extract of the 50 per cent alcohol insoluble residues from *fraction 2*, finally precipitated by four volumes of ethanol.

Fraction 4. Residue remaining after water extraction in *fraction 3*.

Fraction 5. An aliquot of *fraction 3* re-extracted with water and reprecipitated by making the concentration of alcohol 50 per cent.

Fraction 2 on the basis of total solids per mouse uterine unit was found to represent a fivefold concentration of the gonadotropic activity and a lowering in toxicity

to one-fourth that of the original powder. This purification was accomplished with a yield of more than 65 per cent of the gonadotropic activity. *Fraction* 3 exhibits the same toxicity on a unit equivalent basis as the unfractionated extract, but there was a 1.6-fold concentration of the toxicity on a weight basis. *Fraction* 4 was more toxic than the starting powder or than *fraction* 3 on a weight basis, but not as toxic as either of them on a unit equivalent basis. *Fraction* 5 represented as much toxicity on a weight basis as the original powder and was very low in, or free from gonadotropic activity.

Thus it was shown that the toxicity and gonadotropic activity can be separated into two well-defined fractions by the 50 per cent ethanol extraction.

Two recovery experiments on the crude alcohol precipitate from postmenopausal urines were carried out because the activity per unit weight is so high that the toxicity factor can be eliminated. Thus a direct comparison of the 50 per cent ethanol extraction followed by reprecipitation with 4 volumes of alcohol, and of the water extrac-

TABLE 9. VOLUME EFFECT

	Mouse uterine weight response to following dilutions			
	A	B	C	D
M.U.U. equivalents	0.75	1.0	1.5	2.0
Mg. equivalents	5.25	7.0	10.5	14.0
Aliquot 1	6.5 (8)	9.9 (8)	23.6 (8)	23.3 (8)
Aliquot 2	7.4 (8)	11.9 (7)	23.2 (8)	26.5 (8)
Aliquot 3	6.4 (8)	9.9 (8)	22.4 (7)	28.4 (8)
Aliquot 4	7.5 (6)	8.0 (8)	22.4 (7)	21.4 (8)
Aliquot 5	7.6 (8)	20.2 (8)	22.6 (8)	28.1 (8)
Aliquot 6	7.4 (8)	14.1 (8)	27.5 (8)	29.2 (8)

Eight mice were started on each level. Numbers in parentheses indicate the animals surviving.

tion and dialysis followed by reprecipitation with 4 volumes of acetone, can be made with the original alcohol precipitate. The results from the first of these experiments showed that the 50 per cent alcohol extraction and the dialysis procedures gave recoveries of 75 and 60 per cent respectively. In the second of these experiments an attempt was made to test the effect of changes in the volume of the 50 per cent ethanol used to extract the crude alcohol precipitate of postmenopausal urine. In table 9 *aliquots 1* and *2* were extracted with 11.5 cc. of 50 per cent ethanol each; *aliquot 1* was precipitated with 4 volumes and *aliquot 2* with 8 volumes of alcohol. *Aliquots* 3 and 4 were extracted with 30 cc. of 50 per cent ethanol each; *aliquot* 3 was precipitated by 4 volumes and *aliquot 4* by 8 volumes of alcohol. *Aliquot 5* was a suspension of the crude powder injected without further treatment, and *aliquot 6* was an aqueous extract of the crude powder precipitated by the addition of 8 volumes of alcohol.

Obviously a two-fold dilution in 50 per cent ethanol used for extraction does not affect the yield of gonadotropin, and precipitation by 8 volumes of ethanol gives essentially the same results as precipitation by 4 volumes. Comparison of the yields by the alcohol extraction method with those in the last two aliquots again shows that the yield by the 50 per cent alcohol method is slightly less than 100 per cent of the total activity whether the latter be based on the reprecipitation of the aqueous extract (*aliquot 6*) or on injection of the suspended powder (*aliquot 5*). The results also suggest that the water extract (*aliquot 6*) does not give as high a response as the suspension of the crude alcohol precipitate. Whether the slight difference was due to incomplete extraction and/or reprecipitation, or whether injection of the suspension

leads to a more gradual release of the gonadotropin because of temporary adsorption of the activity on inert material was not determined.

In table 10 are given the results of an experiment in which the toxic and nontoxic fractions obtained by the 50 per cent alcohol-extraction procedure were recombined as indicated. It is obvious that the toxic fraction in the doses used does not lower the gonadotropin response in the immature mouse. The results suggest instead a possible augmentation. A similar possible augmentation was observed in the toxic fraction

TABLE 10. RECOMBINATION OF GONADOTROPIC AND TOXIC FRACTIONS FROM POSTMENOPAUSAL URINE

Mouse uterine unit equivalents of toxic fraction	Mouse uterine unit equivalents of non-toxic fraction		
	0.75	1.0	1.5
	mg.	mg.	mg.
0	5.1	12.7	17.3
1	5.7	10.2	23.4
2	5.5	15.2	34.0
4	9.9	15.3	26.5

The figures represent the average uterine weight response to combinations of the two fractions expressed as mouse uterine unit equivalents.

from the urine of normal men. It is probable that a very low content of gonadotropin in the toxic fraction is not the cause of the slight augmentation because no gonadotropin activity was detected in the toxic fraction although the equivalent of 6 M.U.U. was injected. However, it is possible that the undetected activity, if present, may increase the response when it is combined with the nontoxic gonadotropin fraction, or that the toxic fraction decreases the rate of absorption and thus causes an augmentation. It is obvious that recovery studies on urinary gonadotropin from normal urine may be particularly involved due to these complications.

Effect of alkaline treatment on activity of alcohol precipitate. Since Levin uses alkali to decompose the tannate and to extract the alcohol precipitate in his modification of this procedure, we tested the effect of alkaline extraction on our 50 per cent alcohol procedures. Five aliquots of the crude alcohol precipitate of pooled urine from normal males were treated as follows.

Aliquot 1. Extracted with water, dialyzed, and precipitated with 4 volumes of acetone plus a trace of sodium acetate.

Aliquot 2. Extracted with dilute alkali (pH 8.5), dialyzed and precipitated with 10 volumes of acetone plus a trace of sodium acetate.

Aliquot 3. Extracted with 50 per cent ethanol (pH 6.3), not dialyzed. Precipitated with 4 volumes of alcohol plus a trace of sodium acetate.

TABLE 11. EFFECT OF ALKALI TREATMENT

	Mouse uterine weight response to following dilutions				
M.U.U. equivalents	A 0.75	B 1.0	C 1.25	D 1.5	E 2.0
Aliquot 1	10.4 (2)	8.5 (2)	Toxic	24.5 (3)	Toxic
Aliquot 2	7.4 (3)	8.8 (1)	18.2 (3)	8.8 (1)	25.7 (4)
Aliquot 3	8.5 (4)	23.2 (3)	24.4 (4)	26.5 (3)	21.7 (4)
Aliquot 4	21.1 (4)	15.0 (3)	13.5 (4)	29.5 (4)	21.5 (1)
Aliquot 5	7.9 (4)	21.1 (4)	16.3 (4)	25.8 (4)	28.7 (4)

Four mice were started on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

Aliquot 4. Extracted with 50 per cent ethanol (pH 6.3), dialyzed, and precipitated with 4 volumes of alcohol plus a trace of sodium acetate.

Aliquot 5. Extracted with 50 per cent ethanol at pH 8.5, dialyzed, and reprecipitated with 4 volumes of ethanol plus a trace of sodium acetate.

The responses to these solutions are given in table 11. The 50 per cent alcohol method (*aliquot 3*) gave the best yield and the alkaline alcohol extraction followed by dialysis (*aliquot 5*) was next best, being better than the alcohol extraction at pH 6.3 (*aliquot 4*) which was also dialyzed. Due to toxicity it is impossible to detect any difference between the aqueous extracts with and without alkali, but the 50 per cent alcohol extraction again resulted in a better yield than when the water extraction followed by dialysis was used. We conclude that the alkaline extraction does not decrease the yield per se, but that it is undesirable in conjunction with the 50 per cent ethanol extraction method because its use makes it necessary to employ dialysis which does decrease the yield.

SUMMARY

By the immature mouse uterine weight response assay method for gonadotropin it was found that:

1. The precipitation of urinary gonadotropic activity by the addition of 4 volumes of alcohol gives higher yields than the tannic acid or the benzoic acid method, but the product from normal human urine is too toxic to permit direct injection.

2. Aqueous extraction of the crude alcohol precipitate followed by dialysis and reprecipitation causes a loss of activity due mainly to the dialysis rather than to the extraction or reprecipitation.

3. Extraction of the crude alcohol precipitate with 50 per cent ethanol and reprecipitation of the activity from the solution by the addition of 4 volumes of alcohol is a more efficient purification method than dialysis from the standpoints of yield of activity and reduction of toxicity.

4. The toxic material which is insoluble in 50 per cent ethanol has no inhibiting effect on urinary gonadotropic activity when the two fractions are mixed and injected.

5. Attempts to purify the toxic material were not successful.

6. Use of weakly alkaline 50 per cent ethanol as the extraction agent did not alter the yield of activity.

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